Pergamon

Int. J. Biochem. Cell Biol. Vol. 29, No. 1, pp. 63–78, 1997

Printed in Great Britain

PII: S1357-2725(96)00120-3

1357-2725/97 $17.00 + 0.00

REVIEW

Transforming Growth Factor βs and Wound Healing

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The Transforming Growth Factor β superfamily (TGFβ) is one of the most complex groups of cytokines with widespread effects on many aspects of growth and development. The TGFβ isoforms and other family members, e.g. Activins and BMPs, have diverse effects in similar physiological situations. TGFβ is involved in the wound healing process. The three mammalian isoforms (TGFβ1, 2 and 3) and recently other family members, e.g. Activin, have been localised in healing wounds. Manipulation of the ratios of TGFβ superfamily members, particularly the ratio of TGFβ1 relative to TGFβ3, reduces scarring and fibrosis. Such manipulations include reducing the levels of TGFβ1/TGFβ2 using neutralising antibodies or preventing the activation of TGFβs. In chronic or impaired wounds the exogenous addition of TGFβ superfamily members accelerates aspects of the healing process. This review summarises evidence for the role of TGFβ superfamily members in wound healing and how modulation of TGFβ levels can prevent scarring and fibrosis. © **1997 Elsevier Science Ltd. All rights reserved**

Keywords: TGFβs Wound healing Fibrosis Scarring Chronic wounds

Int. J. Biochem. Cell Biol. (1997) **29**, 63–78

INTRODUCTION

It has been more than 10 yr since Transforming Growth Factor β (TGFβ) was first described, and in that time, understanding of the biology of TGFβ has increased rapidly. Ironically, this knowledge has also given rise to many more, as yet unanswered, questions concerning TGFβ's role in normal and abnormal growth and development. The effects of TGFβ on various cell types have been elucidated and it is one of the most pleiotropic and complex cytokines. The TGFβ superfamily currently consists of more than 25 molecules, isolated from many species, e.g. man, mice, chickens, *Drosophila melanogaster* and *Xenopus laevis* and encompassing a wide range of functions (Kingsley, 1994a; Kingsley, 1994b; Meno *et al.*, 1996). The main sub-groups of TGFβ-like genes include the TGFβs 1–5, incorporating the three mammalian TGFβ isoforms, 1, 2 and 3, which are 60–80% homologous; Activins and Inhibins, Bone Morphogenetic Proteins (BMPs), and the DVR (dpp and Vg1 related) group consisting of the decapentaplegic (DPP) and 60A subfamilies (for review see Massagué, 1990; Massagué *et al.*, 1994; Lyons *et al.*, 1991; Sporn and Roberts, 1992; Wozney, 1992; Kingsley, 1994a; Kingsley, 1994b). Much of the recent focus has been on the family members predominantly expressed during embryonic development, including the BMPs/Osteogenic Proteins (OPs), Mullerian Inhibiting Substance (MIS), nodal, dorsalin and the most recently described family member, lefty (Meno *et al.*, 1996).

The superfamily is characterised by a conserved carboxy terminal feature consisting of seven cysteine residues, six of which form a rigid cysteine 'knot'. Almost all family members contain this structure. The TGFβ family of proteins are synthesised and secreted as large pro-peptide molecules consisting of three regions; an amino terminal (5′) signalling sequence, a pro- domain and a mature protein carboxy (3′) domain. The variation in family members occurs within all three regions. The

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Received 22 July 1996; accepted 4 September 1996.

mature protein domains form homo- or occasionally heterodimers (Cheifetz *et al.*, 1988; Ogawa *et al.*, 1992) of two 390 amino acid chains. The associated pro-protein region is called the Latency Associated Peptide (LAP). This has three side chains, two of these are asparagine linked mannose-6-phosphate (M-6-P) oligosaccharides (Purchio *et al.*, 1988). In addition, latent TGFβ (LTGFβ) can contain a protein of variable size called the Latent TGFβ Binding Protein (LTBP; Fig. 1). Several LTBPs of varying size (all separate gene products) have been identified (Wakefield *et al.*, 1988; Yin *et al.*, 1995). Cells transfected with the full (signal, pro- and active domain) sequence of TGFβ1 show little secretion unless an LTBP gene is cotransfected (Miyazono *et al.*, 1991; Saharinen *et al.*, 1996) indicating that the large latent complex (TGFβ1 plus the LAP and an LTBP) is a frequently secreted form. It is unclear if the small latent complex (TGFβ1 plus LAP) can be secreted without LTBP, or if extracellular small latent complex results from proteolytic processing of secreted large latent complex or from cell destruction, e.g. platelet degranulation. Extracellularly, TGFβ1 can exist as either the small non-covalently bound latent complex (TGFβ plus the LAP) retained in fibrin clots following its release from degranulating platelets (Grainger *et al.*, 1995b) or as the large latent complex released directly into serum (Roberts, 1996). The total size of the latent TGFβ1 molecule (LTGFβ1) is more than 200 kD and the mature molecule only approx. 25 kD (two 12.5 monomers). Both the LTBP and LAP must be removed before the mature protein can function, therefore activation of TGFβ is a crucial target for biological control of the molecule. The fate of the LAP once cleaved from mature TGFβ has not yet been determined, although it may be degraded by enzymes such as plasmin.

Activation of TGFβ1 has been reported by various methods *in vitro* and considerably fewer *in vivo*. *In vitro* methods include extremes of pH, heat, plasmin (Lyons *et al.*, 1990), deglycosylation, binding to the 450 kD platelet protein thrombospondin (Schultz-Cherry and Murphy-Ulrich, 1993), proteolytic processing by the convertase furin (DuBois *et al.*, 1995), transglutaminase/Factor XIII of the coagulation system (Harpel *et al.*, 1992), steroids such as retinoic acid (Kojima and Rifkin, 1993), and most recently by reactive oxygen species in redox systems (Barcellos-Hoff *et al.*, 1994; Barcellos-Hoff, 1995). *In vivo* the process of activation has not yet been fully established but evidence suggests that plasmin may also activate TGFβ1, under the control of tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA) and the plasminogen activator inhibitors (PAI). Activation *in vivo* may be due in part to binding of the M-6-P residues on the LAP to the M-6-P/IGF-II receptor, which invokes a conformational change of the molecule thus allowing proteolytic cleavage of the active TGFβ out of the latent complex (Dennis and Rifkin, 1991) (Fig. 1). Addition of M-6-P and anti-M-6-PR inhibits the activation of TGFβ *in vitro*. The large latent TGFβ can be sequestered to extracellular matrix proteins through binding of the LTBP, and released by proteolytic cleavage of LTBP at a later time point (Taipale *et al.*, 1994) (Fig. 1). TGFβ can bind to a variety of matrix proteins including biglycan, decorin, fibronectin, collagen IV and α-2-macroglobulin. Much of the serum TGFβ is covalently or non-covalently bound to α-2-macroglobulin, this is therefore a potential clearance mechanism for excess TGFβ (Danielpour and Sporn, 1990; Webb *et al.*, 1994).

TGFβs have three main cellular receptors, Type I Receptor (RI; 65–70 kD) and Type II Receptor (RII; 85–110 kD) are transmembrane serine/threonine kinases, which are the signal transducing receptors required to be present simultaneously for signalling, and Type III Receptor (RIII; betaglycan) is non-signalling, functioning mainly to present TGFβ to RII (Fig. 1). A Type V receptor (400 kD) has been described (O'Grady *et al.*, 1991) but its exact function is unclear. TGFβ1 initially binds to RII, which has a constitutively active kinase. RI then binds to the TGFβ molecule and becomes phosphorylated by RII. RI and RII form a heteromeric complex (probably a heterotetramer) and a signal is generated (Derynck 1994; Wrana *et al.*, 1994) (Fig. 1). As RII acts upstream of RI, these components have been likened to primary receptors and transducers, respectively (Massagué, 1996). RI cannot bind to a free ligand, only a ligand bound to a type II receptor. The BMP receptor system is slightly different in that both RI and RII bind to a ligand with low affinity and only in combination do they achieve high affinity binding (Rosenzweig *et al.*, 1995). Unlike most other growth factors, TGFβs signal via serine threonine kinases as opposed to tyrosine kinases. Phosphorylation of RI is catalysed by the type II

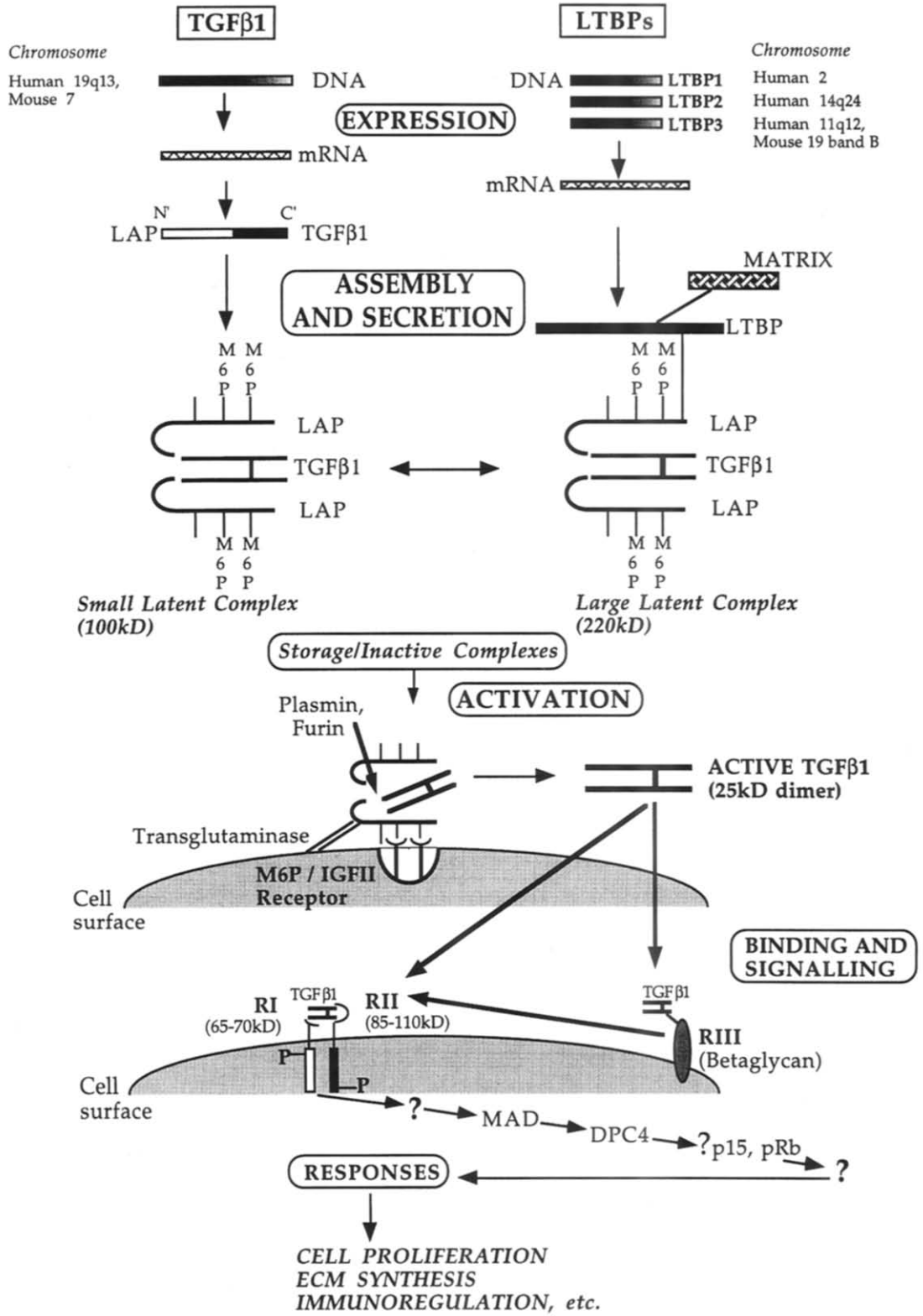

Fig. 1. Summary of the cell biology of TGFβ1.

receptor kinase and occurs in a cluster of five serine and threonine residues in the GS domain, a highly conserved region adjacent to the N terminus of the kinase domain in all type I receptors (Massagué, 1996). The kinase activity of the RII does not appear to be augmented by ligand binding. Mutation of residues in the GS domain of RI either obstructs signalling or produces a constitutively active receptor with elevated kinase activity and the ability to signal a range of responses in the absence of ligand or RII or even in the presence of a dominant negative RII (Wieser *et al.*, 1995). Current evidence suggests that RII cannot generate responses independently of RI. A previous report that overexpression of an inactive RII construct blocked the growth inhibitory effect of TGFβ but not TGFβ's effect on ECM synthesis, was initially interpreted as evidence that RI and RII might each signal separate responses. More likely is the proposal that different TGFβ responses require different strengths of signalling and this may be inhibited to differing degrees by a dominant negative receptor (Feng *et al.*, 1995). Combinatorial signalling via RI and RII allows the response generated to depend on the precise composition of the receptor complex, although our knowledge of which combinations can be induced by specific ligands and the differing nature of the signal and response from each combination is incomplete (Massagué, 1996) but probably important in understanding the differing actions of the various TGFβ isoforms (Shah *et al.*, 1994).

McAllister *et al.* (1994) identified endoglin, the TGFβ binding protein, as the gene for hereditary haemorrhagic telangiectasia (HHT) Type 1 and the disease is characterised by a mutation in the TGFβ receptor coding region. Subsequently, Johnson *et al.* (1996) have shown that HHT Type 2 arises from a mutation in the Activin receptor-like kinase-1 (ALK-1) gene. These data implicate both receptor genes in blood vessel remodelling and therefore suggest important roles in wound healing.

Activins signal using a similar mechanism to the TGFβs, although Activin receptor genes are differentially spliced to give several variants of the Type I and II receptors. Activins are very interesting molecules, which like TGFβs display a wide range of functions, but unlike the TGFβs gain a lot of this diversity from combining as heterodimers. Activins are made up of either a βA or βB chain, so AA, AB and BB forms are formed. The A or B chain can also combine with an α inhibin chain to form the Inhibins A or B (Vale *et al.*, 1990; Mathews, 1994). The TGFβs have not been shown to be as promiscuous in their dimer formation and furthermore multiple Type I or Type II TGFβ receptors have not yet been described. However, receptor specificity/promiscuity for TGFβ isoforms 1, 2 and 3 remains uninvestigated.

Until recently there was very little known about the actual signal transduction molecules responsible for propagating TGFβ's effects. The retinoblastoma gene product Rb and the molecule p15, both tumour suppressor proteins, were recently implicated as signalling molecules for TGFβ1 (Alexandrow and Moses, 1995) and a mouse protein kinase named TAK1 with homology to the mitogen activated protein kinase (MAPKK) family was suggested to participate in the regulation of murine TGFβ1 signalling (Yamaguchi *et al.*, 1995). It has been proposed that a TAK1 binding protein, TAB1, may function as an activator of TAK1 in TGFβ signal transduction (Shibuya *et al.*, 1996). In the last few months, a gene required for Dpp function was cloned from *Drosophila* and named Mothers against Decapentaplegic (Mad) (Sekelsky *et al.*, 1995). It has some similarity to three *Caenorhabditis elegans* sequences, *C. elgans* Mad (CEM or sma) -1, -2 and -3 (Savage *et al.*, 1996). The *Xenopus* homologues (Xmad) were then cloned and analysed by their effect on mesoderm induction in embryonic frogs and found to act downstream of TGFβ family members including BMPs and Activins (Graff *et al.*, 1996; Hoodless *et al.*, 1996). A human Mad protein in the BMP signalling pathway was then identified (Liu *et al.*, 1996) and a related protein, deleted in pancreatic cancer 4 (DPC4) (Hahn *et al.*, 1996) may also belong to this new family of TGFβ signalling molecules (Fig. 1). The most interesting fact about these molecules is that they can produce different ligand-induced signals (e.g. for growth inhibition or ECM synthesis) in response to the strength of signalling (see earlier) and this may explain how TGFβs can have such diverse effects on so many cell types. They appear to be able to sense the type of ligand bound and even the ligand concentration and signal accordingly. These second messenger signalling pathways of TGFβ are exciting and will provide another target for the tissue or cell specific control of TGFβ function.

The initial reports on TGFβ1 described its effects on various cell types; numerous cells produce it and almost all cells respond to it in

some way. Despite the high homology between the three mammalian isoforms (60–80%), TGFβ1, TGFβ2 and TGFβ3 can have different effects on the same cells. The function of the TGFβ1.2 and TGFβ2.3 heterodimers is unclear as they seem to be much less potent than TGFβ1 or TGFβ2 and have much lower binding affinities. The existence of a TGFβ1.3 heterodimer has not yet been reported. Major sources of TGFβ include platelets, leukocytes, bone cells and placental tissue. TGFβ1 can be both inhibitory and stimulatory and is a potent chemoattractant for monocytes, macrophages, lymphocytes, neutrophils and fibroblasts and stimulates the release of cytokines (e.g. Il-1, Il-6, TNFα, bFGF) from these cells (Roberts and Sporn, 1990). TGFβ1 also autoinduces its own expression and the expression of other TGFβ isoforms, so amplifying these effects. TGFβ1 is angiogenic and a recent report suggests that the Type II receptor mediates the antiproliferative effect of TGFβ1 while the Type I receptor mediates the matrix response to TGFβ1 in an *in vitro* angiogenesis model (Sankar *et al.*, 1996), although as discussed earlier this might also be explained by different responses to different strengths of signal. Another group provides evidence that TGFβ1 induces angiogenesis *in vivo* with a threshold pattern and this effect is secondary to its chemoattractant role (Fajardo *et al.*, 1996). TGFβ1 is an important regulator of the extracellular matrix (ECM), stimulating fibroplasia and collagen deposition, inhibiting ECM degrading proteases and upregulating the synthesis of protease inhibitors. Since all these processes are integral to wound healing, the role of TGFβs in wound healing and regulation of their activity are of major clinical significance.

TGFβ AND WOUND HEALING: LOCALISATION AND MODULATION

*Localisation of TGF*β

There have been several reports on the location of TGFβs in wounds *in vivo*, mainly in cutaneous wounds. However, other, non-dermal sites have been the focus of much recent attention, particularly TGFβs in the eye, central nervous system, mouth and skeletal tissue. TGFβs 1, 2 and 3 have been the main topics of investigation but recently Activins and BMPs have been localised during wound healing. The use of novel transgenic technology to create mice deficient in or overexpressing certain TGFβs provides scope for even more research. Models used to examine TGFβ in wounds range through mice, rats, pigs, guinea pigs, rabbits and man. Models of both normal and impaired healing have been studied and the wounding processes also vary through incisional, partial thickness, full thickness, excisional, acute and chronic wounds. The effect of adding exogenous substances to modulate TGFβ during wound healing is dependent on a number of factors such as the dose, the delivery vehicle and the timing of delivery. A review by Roberts (1996) summarised the methods and results in studies determining the activity and efficacy of TGFβ in animal models of normal and impaired wound healing. *In vitro*, the three isoforms are quite similar in their effects, yet *in vivo*, the spatial and temporal distribution and actions of these isoforms is quite specific. TGFβ1 is most abundant in all tissues and cells, then TGFβ2 and least of all, TGFβ3. TGFβ2 is present mostly in bodily fluids such as saliva, amniotic fluid, breast milk and the eye whereas TGFβ3 is not present at all in great amounts in either fluids or tissues (Roberts, 1996).

There are some differences in the spatial expression of TGFβ in different species as determined by immunocytochemistry and *in situ* hybridisation studies, and indeed probably also within species, for example, there are probably subtle strain differences in animals such as mice. In normal skin TGFβ1 displays the least heterogeneity amongst species: it is located in the keratinocytes in the epidermis and capillaries. TGFβ1 is present throughout the basal lamina and mesenchymal cells of all species, but in pig skin TGFβ1 is not present in the intact dermis (Levine *et al.*, 1993) and in human skin, expression occurs in suprabasal keratinocytes (Kane *et al.*, 1991; Schmid *et al.*, 1993a). TGFβ2 expression in normal murine skin is confined to the outer epidermal layers and in pig skin there is strong immunocytochemical staining in all epidermal layers (Levine *et al.*, 1993). In human skin it was reported that there was no TGFβ2 in the dermis of normal, unwounded skin (Schmid *et al.*, 1993a) but other evidence from our laboratory suggests immunocytochemical staining in the suprabasal layer. In rat skin TGFβ2 is associated with hair follicles and sebaceous glands. TGFβ3 is the most variable of the three isoforms: it is constitutively expressed in human epidermal keratinocytes (Schmid *et al.*, 1993a), and it is expressed very strongly in pig skin in both keratinocytes and sebaceous glands (Levine *et al.*, 1993) whereas

in murine and rat skin TGFβ3 is expressed at very low levels in the unwounded dermis (Frank *et al.*, 1996).

Following wounding, the levels of TGFβs alter significantly. In adult mouse incisional wounds, work in our laoratory has demonstrated that the levels of TGFβ1 and 2 increase rapidly at 1 day post-wounding but TGFβ3 only increases a few days post-wounding, when TGFβ1 levels are decreasing. In transgenic mice heterozygous for the TGFβ3 null mutation and hence with 50% less TGFβ3, (Proetzel *et al.*, 1995), the level of TGFβ1 increased more markedly than in homozygote control mice 1 day following incisional wounding, while the level of TGFβ2 decreased at 1 day and then increased at 7 days post-wounding. Another study from our laboratory using a transgenic mouse, which overexpresses TGFβ1 under the control of an albumin promoter (Sanderson *et al.*, 1995), found that the level of TGFβ3 was increased and TGFβ1 decreased in an incisional wound 7 days post-wounding, compared to wounds in control mice. These results suggest there is a cross regulation of the TGFβ1 and TGFβ3 isoforms during wound healing; when TGFβ3 is high, TGFβ1 is low and vice versa. Where the ratio of TGFβ3 to TGFβ1 is high wounds heal with improved scarring (see later). In the TGFβ1 knockout mouse, the main phenotype is a progressive wasting disorder and concomitant multifocal inflammation, which results in death at approx. 3 weeks after birth (Shull *et al.*, 1992; Kulkarni *et al.*, 1993). Wound healing studies were therefore performed on 10-day-old mice and the wounds harvested after another 10 days and analysed (Brown *et al.*, 1995). There were no differences in wound closure, nor in the relative amounts of TGFβ2, PDGF-A or B, or TNFα. There was a characteristic inflammation in the wounds, along with slightly decreased granulation tissue formation, vascularity, collagen deposition and re-epithelialisation in the knockout animals. Compared to wounds in control animals, TGFβ1 was absent but TGFβ3 was increased (Shah, Roberts *et al.*, unpublished) and the wounds healed with minimal scarring.

Work in our laboratory has investigated the profile of TGFβs in both normal rat skin and in incisional dermal wounds. TGFβ1 increased rapidly in the wound at 1 hr post-wounding, and had decreased by 48 hr, but with persistent immunoreactivity in the blood vessels at the base of the wound. A second peak of TGFβ1 was observed at 7 days post-wounding. TGFβ2 increased at 8 hr post-wounding and decreased again by 72 hr. TGFβ3 immunoreactivity was increased in the clot after 1 hr and remained upregulated in the wound until 14 days post-wounding.

In a study by Levine *et al.* (1993), the effect of dermatome induced wounding on the levels of immunoreactive TGFβ in pig skin was determined up to 7 days post-wounding. TGFβ2 and 3 were upregulated by 24 hr following wounding and were present in the epidermis and granulation tissue, the immunostaining subsequently decreased in intensity with time. TGFβ1 was not present except in hair follicles until 5 days post-wounding, following re-epithelialisation of the wounds, suggesting that in pig skin, TGFβ2 and TGFβ3 are important in wound closure with TGFβ1 playing a lesser role. However, Kane *et al.* (1991) reported that, in a similar dermatome-excised porcine wound, there were low basal levels of TGFβ1 protein in the unwounded skin and this increased significantly at 24 hr post-injury and then decreased to normal levels by day 7.

In rabbits, Nath *et al.* (1994) performed surgery simultaneously on both fetuses and adult mothers. The superficial wounds on the adult were incisions on the dorsal surface, which were sutured. The levels of TGFβ proteins were assessed by immunocytochemistry after 3, 5 and 7 days. After 3 or 5 days there were no changes in the basal expression of TGFβ1 or TGFβ2, but at 7 days post-wounding, both had increased with TGFβ1 the predominant isoform. Unfortunately, sex steroids are known to affect TGFβ expression (Jeng *et al.*, 1993; Grainger and Metcalfe, 1996), which complicates this experiment on pregnant female adults as opposed to male adults, which are normally used for wound healing studies.

Frank *et al.* (1996) described the regulation of the three TGFβ isoforms and their receptors in normal and impaired murine excisional wound healing. TGFβ1 mRNA levels increased ninefold within 24 hr, and remained elevated for several days, TGFβ2 mRNA did not increase until 5 days post-wounding and only increased by approx. 4.5-fold, whereas TGFβ3 was not expressed until 5 days, reaching a maximum at 7 days (12.5-fold induction). High levels of TGFβRII mRNA were expressed in normal skin, lower levels of TGFβRI and both were upregulated maximally at 1 day post-wounding

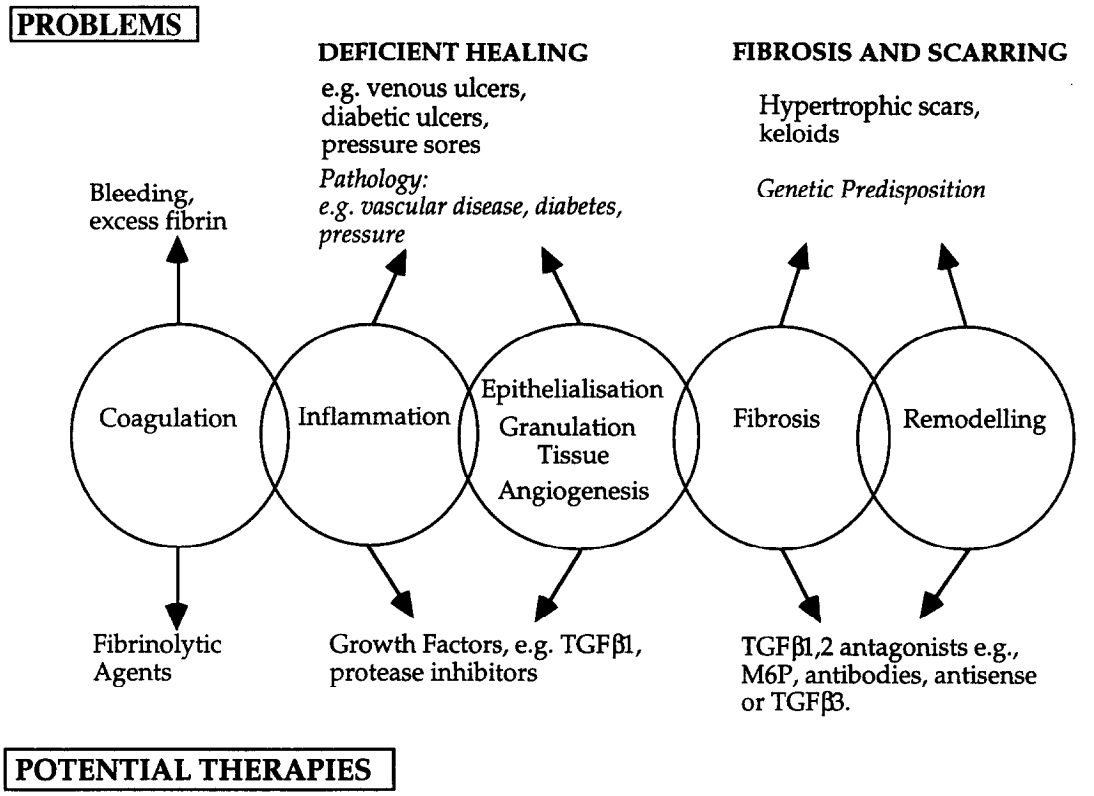

Fig. 2. Major phases of wound healing, their associated pathologies and potential therapies.

and remained elevated through to 14 days. In glucocorticoid treated, thus impaired wounds, TGFβ1 and TGFβ2 expression was inhibited and TGFβ3 expression induced earlier than in control wounds. The dexamethasone treatment increased the level of TGFβRI and decreased TGFβRII in the impaired wounds. Demonstration of decreased levels of TGFβ1 and TGFβ2 in impaired wound healing supports the use of exogenous TGFβs in the treatment of healing impaired wounds (Pierce *et al.*, 1989; Beck *et al.*, 1993). Interestingly the selective effect of dexamethasone on increasing the ratio of TGFβ3 relative to TGFβ1 may be important in the anti-inflammatory and scar improving properties of glucocorticoids in wound healing. Hubner *et al.* (1996), from the same laboratory, investigated the expression of Activin and Activin receptors in healing excisional wounds. Activins, inhibins, the binding protein follistatin and Activin receptors have been shown to be present at different levels in normal skin. A strong induction of Activin A and B mRNA expression at 1 day post-wounding was observed, which persisted for up to 7 days in the case of Activin A and 13 days for Activin B; the receptor levels were unaffected. These results suggest that Activins may be important in normal wound repair. We have shown that exogenous addition of recombinant Activin A to rat incisional wounds accelerates re-epithelialisation up to 7 days post-wounding and also improves the final scar quality.

Another type of impaired wound is that seen in the aged, who seem to heal their wounds more slowly than the young. Investigation of both mRNA and protein levels of TGFβs and other cytokines following incisional wounding in young, middle aged and old mice, and humans of defined health status has been carried out in this laboratory (Ashcroft *et al.*, 1995). In general TGFβ1 levels were reduced in the old compared to the young, but at 14 days post-wounding, the level of TGFβ3 increased in the old and remained higher thereafter compared to the young. The final scar appearance was better in the old than in the young again suggesting that high levels of TGFβ3 relative to TGFβ1 are an important regulator of scar quality.

The majority of wound healing investigations are performed on acute dermal wounds. Schmid *et al.* (1993a) determined the pattern of TGFβ and TGFβRII mRNA expression by *in situ* hybridisation in biopsies from both acute (burn) and chronic (decubitus ulcer) human

wounds. TGFβ3 is the constitutively expressed isoform in human skin, TGFβ1 and TGFβ2 are apparently not present at high levels. In both the acute and chronic wounds analysed, the level of TGFβ3 and TGFβRII was high. In the acute wound, TGFβ1 was upregulated in the migrating keratinocytes and hair follicles but remained low in chronic wounds. This suggests that the lack of TGFβ1 in the chronic wounds is involved in their failure to heal.

MANIPULATION OF TGFβ LEVELS IN WOUNDS: ACCELERATION OF HEALING, ANTI-SCARRING AND ANTI-FIBROTIC THERAPIES (Fig. 2)

It is clear from studies on TGFβ deficient mice and analysis of the cytokine profile of chronic wounds, that TGFβ is necessary for wound healing. Studies where TGFβ was added to wounds, shortly after its discovery, reported an acceleration in healing. Sporn *et al.* (1983) injected purified bovine TGFβ into wire mesh chambers in the backs of rats and observed an accumulation of total protein, collagen and DNA. Mustoe *et al.* (1987) investigated the effect of exogenous platelet purified TGFβ in the healing of incisional wounds in the backs of male rats and reported an increase in breaking strength of 220% after only 5 days, and healing appeared to be accelerated by approx. 3 days. McGee *et al.* (1989) also reported that a single application of recombinant TGFβ1 accelerated incisional wound healing and increased tensile strength in a rat wound. Quaglino *et al.* (1990, 1991) described the effects of TGFβ1 on healing in excisional and also incisional wounds in a porcine model. Granulation tissue formation was increased at 6 days post-wounding in the incisional wounds and *in situ* hybridisation studies revealed an upregulation of expression of ECM proteins, a decrease in the expression of the ECM degrading enzyme stromelysin and an auto-induction of TGFβ1 mRNA. These early studies mainly focused on TGFβ1, but another study examined the expression of all three TGFβs during repair following exogenous addition of each isoform (Schmid *et al.*, 1993b). Partial thickness thermal wounds were created on the backs of young and old mice, TGFβ1, 2, or 3 was added topically and the skin processed up to 3 days post-injury. The addition of each isoform appeared to increase expression of only TGFβ1. In the old mice, the basal levels of TGFβ1 in the wounds were decreased, but reversed by topical application of TGFβ1. Salomon *et al.* (1990) had previously reported that in doxorubicin (adriamycin) treated animals, application of TGFβ1 also reversed the wound healing deficit. These results therefore suggested that exogenous TGFβ1 could accelerate healing not only in normal but also in impaired wounds.

Impairment of the healing process can occur as a consequence of chemotherapy, metabolic disorders, ageing, vascular disease, pressure or radiation therapy. Numerous studies have shown that supplementation with TGFβ1 accelerates healing in impaired wounds. Beck *et al.* (1993) gave one dose of recombinant human TGFβ1 systemically to rats that had impaired healing. There were two groups, aged rats and glucocorticoid treated rats. The single dose increased incisional wound breaking strength to levels similar to normal young rats. Steroids reduce the numbers of inflammatory cells and macrophages in a wound, so the addition of TGFβ1 probably acts on the fibroblasts to increase collagen deposition and therefore tensile strength. It was noted that although the circulatory half-life of TGFβ1 was less than 5 min, the same effect could be achieved whether TGFβ1 was given 24 hr before wounding, at the time of wounding or 4 hr after wounding. This group also demonstrated that repeated injection of TGFβ1 reversed steroid impaired healing, but observed that at a distant injection site (technically another wound), there was a fibrotic response. This is similar to the results described later, that repeated TGFβ1 can cause fibrosis. Other studies describe the effects of exogenous, natural or recombinant TGFβ2 in the wound healing response of aged animals (Cox *et al.*, 1992). Apart from the increase in cell recruitment, ECM deposition and acceleration of granulation tissue formation, TGFβ2 had a beneficial effect on the wound microvasculature.

Non-healing human chronic wounds, whatever the cause (Fig. 2), whether diabetic, decubitus or venous are a significant clinical problem, so the rationale behind using animal models of impaired healing is to test the efficacy of TGFβs for clinical application. Higley *et al.* (1995) described the localisation of TGFβ1 and TGFβ2 in biopsies from patients with venous ulcers. There was less immunoreactive TGFβ2 in both the control wounds and ulcers. Venous ulcers have characteristic extracellular matrix 'cuffs' around blood vessels (Herrick *et al.*,

1992). There are high levels of TGFβ1 bound to these blood vessel cuffs in venous ulcers, but very little in the wound bed, whereas in normal wounds, TGFβ1 is localised in the granulation tissue but absent in blood vessel cuffs. Distribution of TGFβs in chronic wounds may be more important than total amount.

A clinical trial using bovine derived TGFβ2 in the treatment of venous stasis ulcers provided initially encouraging data (Robson *et al.*, 1995). The total ulcer area was reduced and showed that since TGFβ2 was not detrimental to healing, it may be useful in accelerating healing of other chronic wounds. TGFβ2 has been used successfully in the retina of the eye to treat macular holes (Glaser *et al.*, 1992). However, despite early encouraging results, subsequent studies of exogenous addition of growth factors to chronic human wounds have been disappointing. There are many reasons for this. The mode of delivery is very important—chronic wounds have high levels of proteases, so topical application in the absence of a protease inhibitor is unlikely to yield pharmacological doses to the target cells deep in the wound. The receptor status of chronic wounds is often unknown and variable. The underlying disease, e.g. vascular pathology, needs to be corrected and there may be marked variation in oxygen tension within the tissue. The specific combination of growth factors required at any time to stimulate the healing of a chronic wound may vary. Most significantly, nearly all human trials of chronic wound healing agents show a significant placebo effect with the control arm healing well and so reducing the possibility of demonstrating a significant therapeutic effect from the exogenous agent.

Reduction of scarring

Fibrosis and scarring post-wounding are serious clinical problems (Fig. 2), with few known treatments. Scarring can cause adverse cosmesis, loss of function, e.g. strictures and adhesions following burns and abdominal surgery, and restriction of growth in children. It has adverse consequences in nearly every organ and tissue, e.g. glial scarring in the CNS, scarring in muscle, joints, skin, heart, etc. Cutaneous scarring may be defined as the macroscopic disturbance of the normal structure and function of the skin, arising as a consequence of wound healing, and owing to the changes in epidermal, dermal and subcutaneous tissue at the time of wounding (Ferguson *et al.*, 1996). Adult dermal scars will remodel for up to 1 yr following wounding. An important discovery was the observation that embryonic or early fetal wounds heal without scars (Ferguson *et al.*, 1996). This is dependent on a number of factors such as gestational age, position of the wound on the body of the embryo, species, etc. (for review see McCallion and Ferguson, 1996; Ferguson *et al.*, 1996; Shah *et al.*, 1996). Differences between fetal and adult wound healing have been documented: important in the scar free phenotype are the reduced fetal inflammatory response and hence altered cytokine profile, reduced fetal wound angiogenesis and absence of fetal wound fibrin clots (Whitby and Ferguson, 1991a; Whitby and Ferguson, 1991b). The warm, sterile enviroment of the fetus, excessive hyaluronic acid deposition and intrinsic differences in developing tissues are not the most important determinants of fetal scar-free healing (Ferguson *et al.*, 1996). These observations of differences in fetal scar free versus adult scar forming healing lead to experiments in adult wound healing to attempt to improve/eliminate scarring. Initial experiments focussed on TGFβs as there are profound differences between the embryo and the adult and TGFβ1 regulates many important events in tissue fibrosis.

Initially we demonstrated that intradermal injection of a polyclonal neutralising antibody to TGFβ1,2 significantly reduced scarring from a rat incisional wound (Shah *et al.*, 1992; Shah *et al.*, 1994). Neutralisation of both TGFβ1 and TGFβ2 is required and the antibodies must be applied at the time of or shortly after wounding to reduce the amplification cascade of TGFβ1 at the wound site (Shah *et al.*, 1992; Shah *et al.*, 1994; Shah *et al.*, 1995). Neutralisation of TGFβ1,2 reduced the inflammatory and angiogenic responses and altered the deposition of ECM, without reducing the tensile strength of the wound. The reformed wound dermis had a similar histological organisation as control skin, so resulting in an absent scar. Interestingly exogenous addition of TGFβ3 also markedly improved scarring with a restitution of the neodermis, a decrease in inflammatory cells and early fibronectin, but an increase in angiogenesis (Shah *et al.*, 1994). Preliminary evidence suggests that TGFβ3 downregulates the production of TGFβ1 (and vice versa). This together with the possibility that TGFβ3 interacts with a different receptor subset than TGFβ1 may explain its unique antiscarring

activity. Therefore, limiting scarring depends on the ratio of TGFβ3 relative to TGFβ1/TGFβ2 early in the wound healing cascade. Prevention of activation of TGFβ1 by competitive inhibition of binding of the LAP at the M-6-P/IGFII receptor using exogenous M-6-P has a marked antiscarring effect in rodent, pig and human wounds (McCallion and Ferguson, 1996). Exogenous M-6-P also reduces scarring in the eye following surgery (Sutton *et al.*, 1996) whilst antibodies to TGFβ1 or decorin protein both decrease scarring in the central nervous system following experimental surgical lesions (Logan *et al.*, 1994). Decorin binds and neutralises all three TGFβ isoforms (Hildebrand *et al.*, 1994) whilst it is also believed that all three TGFβ isoforms require the M-6-P receptor for activation. However, early in wounding the predominant TGFβ isoform present is TGFβ1 released from degranulating platelets and so both M-6-P and decorin may reduce scarring by preventing the activation of (predominantly) TGFβ1. This suggests that the antiscarring effects of decreasing the ratios of TGFβ1/TGFβ2 relative to TGFβ3 may be generalisable to all body tissues/organs and indeed to pathological fibrotic states.

TGFβ *and fibrosis*

Excessive fibroplasia and extracellular matrix deposition in tissues is the pathological condition known as fibrosis. Exogenous TGFβ induces fibrosis and angiogenesis when injected subcutaneously into newborn mice (Roberts *et al.*, 1986). Krummel *et al.* (1988) demonstrated a similar effect in a fetal rabbit model when subcutaneous sponge implants containing TGFβ1 were analysed 7 days post-wounding and found to contain large numbers of inflammatory cells and fibroblasts with marked collagen deposition. There have been numerous investigations into the role of TGFβs in fibrosis, and TGFβ1 has been implicated in the pathogenesis of kidney, liver, lung, skin, arterial, neural and arthritic fibrosis in both animal and human models (reviewed in Border and Noble, 1994).

In the lung, the expression of TGFβ1 has been investigated in experimental bleomycin induced fibrosis in rats and mice and found to be upregulated (Westergren-Thorsson *et al.*, 1993; Hoyt and Lazo, 1988), with macrophages the primary source. In a silica-induced fibrotic rodent model, TGFβ1 expression displayed a temporal and spatial correlation with Type 1 procollagen expression (Mariani *et al.*, 1996). In a clinical situation, increased TGFβ1 is associated with pulmonary fibrosis: immunocytochemical and *in situ* localisation of TGFβ1 was elevated adjacent to fibroblasts actively synthesising collagen (Broekelmann *et al.*, 1991; Khalil *et al.*, 1991, Corrin *et al.*, 1994). Recently it has been reported that TGFβ1 alone, not TGFβ2 or TGFβ3 is upregulated in the epithelial cells of advanced human pulmonary fibrosis (Khalil *et al.*, 1996). By contrast, Santana *et al.* (1995) reported that all three TGFβ isoforms were induced in rat lungs following bleomycin instillation. Matsuse *et al.* (1995) demonstrated that another TGFβ family member, Activin A is present in both normal and bleomycin treated lungs, but significantly increased in the latter. Giri *et al.* (1993) found that treating mice with an antibody to TGFβ1 or a combination of anti-TGFβ1 and anti-TGFβ2 5 min after intratracheal bleomycin significantly reduced the fibrotic reaction. BCG-induced murine lung fibrosis also abated following anti-TGFβ1 administration (Denis, 1994). Therapeutic regimens for lung fibrosis such as neutralising TGFβ1 or preventing its activation are discussed by McAnulty and Laurent (1995).

Glomerular fibrosis, whether acute or chronic glomerulonephritis, diabetic nephropathy or induced nephrosis, has been investigated with regard to the involvement of TGFβ1 (reviewed in Border and Noble, 1994). In this condition there is also a close correlation between TGFβ1 synthesis and progression of the disease (Okuda *et al.*, 1990; Bollineni and Reddi, 1993; Yamamoto *et al.*, 1994). Neutralization with antiserum halted extracellular matrix accumulation (Border *et al.*, 1990) but sustained delivery of antiserum resulted in persistent TGFβ1 expression (Yamamoto *et al.*, 1994), which exacerbated the condition. Insulin treatment of diabetic rats reduced the production of TGFβ1 (Yamamoto *et al.*, 1993) and *in vitro*, glucose and angiotensin-II increased the levels of TGFβ1, fibronectin, collagen and proteoglycan (Kagami *et al.*, 1994; Ziyadeh *et al.*, 1994). Isaka *et al.* (1996) described how genetic transfer of the proteoglycan and TGFβ binding protein, decorin, into the gluteal muscle of rats increased the endogenous muscle and glomerular levels of decorin significantly. The level of TGFβ1 was substantially reduced but not eliminated, and fibrosis decreased, implying that decorin could be a novel therapeutic approach

to combat TGFβ mediated fibrosis of the kidney. Another novel anti-fibrotic therapy is to use the TGFβ family member OP-1 (BMP-7). Evidence from OP-1 knockout animals that have undeveloped kidneys and die as a result, implicate the OP-1 gene in kidney development (Luo *et al.*, 1995) and it has been suggested that exogenous OP-1 given to rats with damaged kidneys may alleviate the symptoms of fibrosis (Coghlan, 1996).

In the pathology of the established disease muscular dystrophy, TGFβ1 levels are elevated. Duchenne muscular dystrophy, which is characterised by muscle wasting because of myofiber degeneration and fibrosis, had significantly higher TGFβ1 levels than control or other dystrophic conditions (Bernasconi *et al.*, 1995). Fibrotic skin conditions often arise as a consequence of impaired healing or systemic sclerosis. Grainger *et al.* (1995a) reported that the serum concentration of active TGFβ is decreased in atherosclerosis while in systemic sclerosis (SSc), active TGFβ1 was detectable in only some patients (Snowden *et al.*, 1994). In another study, the bronchial lavage fluid (BAL) from SSc patients exhibited a differential increase in TGFβ1 and decrease in TGFβ2 (Ludwicka *et al.*, 1995). TGFβ1 also appears to induce *de novo* synthesis of Type VII collagen in the dermis of SSc patients (Rudnicka *et al.*, 1994). Other fibrotic lesions in the skin, particularly keloids with characteristic excessive deposition of collagen or scars resulting from burns have also been analysed with respect to TGFβ. Russell *et al.* (1988) investigated the response of normal and keloid-derived fibroblasts to TGFβ and EGF and observed a difference in the stimulation of thymidine incorporation. TGFβ augmented the stimulatory effect of EGF in keloid fibroblasts and decreased the effect of EGF in normal fibroblasts. Peltonen *et al.* (1991) reported the colocalisation of Types I and VI collagen and TGFβ1 in keloids. Tuan *et al.* (1996) used a 3-D gel system to determine the levels of uPA and PAI-1 in normal and keloid human fibroblasts and reported a reversal of the ratio of uPA and PAI-1 in keloid fibroblasts, a similar pattern was observed in normal cells treated with TGFβ1.

Liver fibrosis is yet another condition where TGFβ1 plays a central role in the pathology, closely correlated with increased collagen expression (Nagy *et al.*, 1991), decreased hepatic cell degeneration and the induction of apoptosis (Takiya *et al.*, 1995). Williams and Knapton (1996) investigated the expression of TGFβ1, TGFβ2, TGFβRII and the immunolocalisation of TGFβ1 in quartz-induced hamster and murine hepatic fibrosis, cirrhosis, adenoma and carcinoma and found a strong temporal and spatial pattern. Transgenic mice, which overexpressed TGFβ1 in hepatocytes under the control of an albumin promoter, had multiple fibrotic lesions in the liver, glomerulonephritis, arteritis and myocarditis and changes in the pancreas and testes, as well as increased apoptosis of the hepatic cells (Sanderson *et al.*, 1995). Bottinger *et al.* (1996) have shown that recombinant TGFβ1 LAP is a potent inhibitor of the activities of TGFβ1, TGFβ2 and TGFβ3 and can reverse the fibrotic effect of elevated TGFβ1 in these mice, suggesting a potential use for recombinant LAP in the prevention of fibrosis. Myelofibrosis (Martyre, 1995), otosclerosis (Bodo *et al.*, 1995), restenosis (Nikol *et al.*, 1992) and vitreoretinopathy (Connor *et al.*, 1989) are other conditions where there is strong evidence for the involvement of TGFβ1.

There is an increasing literature concerning TGFβ1 and fibrosis and many of the experimental anti-fibrotic therapies being devised are targeted at altering the levels of the TGFβs, particularly at attenuating TGFβ1 production and activation in the various systems.

CONCLUSIONS

TGFβ superfamily molecules play a crucial role in wound healing. Most data have been accumulated for TGFβ1 but it is clear that other isoforms, e.g. TGFβ3, and family members, e.g. Activin and BMPs, are also involved. Emerging data on the role of other superfamily members together with new information on activation mechanisms, receptor specificity and second messenger signalling pathways all provide new targets for experimental and therapeutic manipulations. Exogenous addition of TGFβ superfamily members may be useful to stimulate certain chronic healing states or to protect against high doses of chemotherapy or radiation. Conversely, modulation of the profile of TGFβ superfamily members, e.g. by elevating the levels of TGFβ3 relative to TGFβ1/TGFβ2, may be important in the prevention of scarring and fibrosis.

Acknowledgements—Research summarised in this paper has been supported by grants from the Medical Research

Council, The Wellcome Trust and Johnson and Johnson, for which we are grateful.

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